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Biodiesel from Aquatic Species

Project Report: FY 1993

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PREFACE

This report summarizes the progress and research accomplishments of the Biodiesel from Aquatic Species Project, field managed by the National Renewable Energy Laboratory (NREL), through September 1993. The project receives its funding through the Biofuels Systems Division of the U.S. Department of Energy.

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I. PROJECT OVERVIEW

INTRODUCTION

The development of alternative, renewable sources of liquid transportation fuels will be vital to meeting future domestic energy needs and will help to facilitate compliance with new clean air standards. It is expected that gasoline production in the United States will be reduced as petroleum feedstock availability becomes more limited and alcohol-based fuel production increases. Because diesel fuel is a coproduct of the gasoline refining process, the future reduction in gasoline production requires the development of a substitute, renewable source of diesel fuel. An extremely attractive candidate to fulfill this need for a diesel fuel substitute is "biodiesel." Biodiesel is defined as the esters derived from oils and fats from renewable biological sources (Clements 1992). Currently, the primary sources are oilseeds (such as soybean and rapeseed) and animal fats.

Biodiesel is a cleaner fuel than petroleum diesel. It is virtually free of sulfur, thereby eliminating the production of sulfur oxides during combustion. Emissions of hydrocarbons, carbon monoxide, and particulates during combustion are also significantly reduced in comparison to emissions from petroleum diesel. These properties make biodiesel useful in facilitating compliance with the Clean Air Act Amendments of 1990 (CAAA). An additional benefit of biodiesel is that it is thought to be a nontoxic, biodegradable fuel that can be used in standard, unmodified diesel engines. Biodiesel provides essentially the same energy content and power output as petroleum-based diesel fuel while reducing emissions. Biodiesel's low pollutant emissions can be extremely useful in Environmental Protection Agency (EPA) non-attainment areas, which are typically central cities with acute local air pollution problems. Buses and other fleet vehicles running on biodiesel have the potential to make a major impact in these markets. In addition, biodiesel may be useful as an enhancer of conventional petroleum diesel, as it could be used to reduce aromatics and increase the cetane number (equivalent to octane in gasoline) to mandated levels when added to petroleum diesel. Using biodiesel as a fuel enhancer would generate a sizeable market similar to that for methyl tertiary butyl ether (MTBE) and other oxygenates used in gasoline; it should be noted that the market price of fuel enhancers such as MTBE is almost twice that of gasoline (Shay 1993).

The CAAA provide significant opportunity for the replacement of conventional diesel fuel with biodiesel. In 1993, clean diesel fuel regulations take effect, requiring the sulfur content of diesel used in highway transport trucks to be limited to 0.05%, for a reduction of 90%. This requirement will affect roughly 80% of the distillate fuel consumed in the transportation sector (Energy Information Administration 1993). Assuming that the percentage of diesel fuel affected remains constant (i.e., 80%), the reformulation is projected to affect 24.1 billion gallons (91.2 billion liters) in 1995, 26.5 billion gallons (100 billion liters) in 2000, 29.1 billion gallons (110 billion liters) in 2005 and 31.3 billion gallons (118 billion liters) in 2010 (Energy Information Administration 1993).

Although these regulations currently apply only to highway motor fuels, the refinery industry expects that they will be extended to all distillate fuels in the near future (Cambridge Energy Research Associates 1992). If these standards were extended to all distillate fuels, the potential market for biodiesel would roughly double to upwards of 60 billion gallons (227 billion liters) per year (7.8 quadBtu/yr [8.2 EJ/yr])[1]. Inasmuch as the total vegetable oil and tallow supply in the United States (Commodity Research Bureau 1992, United States Department of Agriculture 1992) is currently equivalent to less than 3 billion gallons (11 billion liters) of biodiesel fuel (0.35 quadBtu/yr [0.37 EJ/yr]) the vegetable oil and tallow supply is insufficient for the fuel market (Fig. 1). The current supply is needed for food and other uses. Assuming the current supply could be increased by 50% and that amount (1.5 billion gallons [5.7 billion liters] = 0.17 quadBtu/yr [0.18 EJ/yr}) could be devoted to fuel use, then only 2% of the market demand could be supplied by biodiesel from oilseeds. Even that number is not certain because there will be other energy crops competing with oilseeds for land and water resources, such as lignocellulosics for fuel ethanol production (Lynd et al. 1991). A recent estimate for a 13-state region in the southeastern United States indicates that 0.64 billion gallons (2.4 billion liters; 0.07 quadBtu/yr [0.07 EJ/yr]) of biodiesel could be produced (Raymer and Van Dyne 1992). On a global basis the total production of plant and tropical oils, animal fats, and marine oils is only about 20 billion gallons (76 billion liters; 2.6 quadBtu/yr [2.7 EJ/yr]), or about one-third of the projected demand for diesel fuel in the United States. The global supply is for the most part unavailable for use as biodiesel, as it supplies food and other markets.

Although the small amount of biodiesel from oilseeds and tallow provides an opportunity to satisfy early market demand, the biofuels industry must have another way to meet the need for these large

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[1]quadBtu = quadrillion Btu = 10^{15} Btu; EJ = exajoule = 10^{18} joule

amounts of diesel fuel. Microalgae provide the opportunity to satisfy much of that need without competing with other technologies and sectors for scarce resources. Microalgae are microscopic aquatic plants with the potential to produce large quantities of lipids (plant oils). Biodiesel is ordinarily considered to be derived from oilseeds and animal fats, but a virtually identical biodiesel can be made from microalgal lipids. Microalgal biodiesel would come on line later to fulfill larger market demands from the diesel market, and enhance the long-term potential of biodiesel as a renewable energy source. The primary goal of the Biodiesel from Aquatic Species Project is to develop the technology for growing microalgae as a renewable biomass feedstock for the production of a diesel fuel substitute (biodiesel), thereby reducing the need for imported petroleum. Microalgae are of interest because of their high growth rates and tolerance to varying environmental conditions, and because the lipids they produce can be extracted and converted to substitute petroleum fuels, such as biodiesel.

Microalgae can be grown in arid and semi-arid regions with poor soil quality where woody or herbaceous crops cannot be grown. Saline water from aquifers or the ocean can be used for growing microalgae. Such water has few competing uses and cannot be used for agriculture, forestry, or as potable water. This technology is complementary to ongoing efforts to grow lignocellulosic biomass in areas with good soil and water resources because microalgae are projected to be grown in those areas where lignocellulosics or oilseed crops will not grow well (desert southwestern United States and other areas with poor soils). Also, the yield of biomass per acre from microalgae is three- to fivefold greater than the yield from typical crop plant acreage.

Projected global climate change provides another important rationale for the biodiesel project. Climate change, which has been linked to the accumulation of excess carbon dioxide in the atmosphere, has the potential of producing economic and geopolitical changes with profound impacts on our economy and the energy industry. The burning of fossil fuels, primarily in power plants, is the major contributor to the excess carbon dioxide. Because the primary nutrient for microalgal growth is carbon dioxide, operation of microalgal biomass farms has emerged as a promising candidate in the search for alternative approaches to ameliorate global climate change. The microalgae essentially recycle the carbon dioxide from the power plant's stack gases into a secondary energy product (biodiesel). Although this carbon dioxide is eventually released when the fuel is burned, the process effectively doubles the amount of energy generated for a given quantity of carbon dioxide. Studies have shown that land and saline water are available in areas such as New Mexico and Arizona to support extensive microalgae facilities. The carbon dioxide emissions from all the power plants in these two states could be trapped by microalgae farms covering about 0.25% of the total land area. If this technology is expanded to other states, or projected future capacity is brought on line in Arizona and New Mexico, the farms could supply at least 2 quadBtu (2.1 EJ) of energy, equivalent to more than 50% of the transportation diesel fuel used in the United States.

Studies have shown that economic fuel production will require the microalgae to be grown in intensive culture in large outdoor ponds. The system design consists of 6-in- (0.15m)-deep, raceway-shaped ponds with a paddlewheel for circulating the water. Carbon dioxide (perhaps in the form of power plant stack gases) and other nutrients are injected into the culture to optimize algal growth and oil production. Lipid accumulation is generally triggered by environmental stress, such as depletion of a key nutrient.

Project researchers have collected and studied more than 3000 strains of microalgae from desert and saline environments. From these, a number of promising lipid-producing species have been identified. These organisms grow over a wide range of salinities, produce significant quantities of lipid oils, and achieve growth rates of nearly three doublings per day. Some of these species tolerate temperatures of 100°F (38°C) or higher.

Research is now focused on applying genetic techniques to control the lipid production of microalgae. This effort builds on extensive strain characterization research, as well as on biochemical studies of the metabolic pathways for lipid synthesis. By manipulating culture conditions, scientists can already increase the lipid content of the cell from the 5% to 20% found in nature to more than 60% in the laboratory and more than 40% in outdoor culture. The goal is to develop genetically improved strains of microalgae that produce even higher lipid levels and for which the process of lipid accumulation could be better controlled.

OVERVIEW OF RESEARCH

Researchers in the Biodiesel/Aquatic Species Project focus on the use of microalgae as a feedstock for producing renewable, high-energy liquid fuels. In 1979, the U.S. Department of Energy (DOE) and the National Renewable Energy Laboratory (NREL) initiated the Aquatic Species Project as part of the overall effort in biofuels. The project began to focus exclusively on fuels from microalgae in 1982.

The program's basic premise is that microalgae, which have been called the most productive biochemical factories in the world, can produce up to 30 times more oil per unit of growth area than land plants. It is estimated that 150 to 400 barrels of oil per acre per year (0.06 to 0.16 million liters/hectar) could be produced with microalgal oil technology. Initial commercialization of this technology is envisioned for the desert Southwest because this area provides high solar radiation and offers flat land that has few competing uses (hence low land costs). Similarly, there are large saline aquifers with few competing uses in the region. This water source could provide a suitable, low-cost medium for the growth of many microalgae.

The project has supported research at NREL, as well as in industry, other government laboratories, and universities.

Project Goal

The goal of the Biodiesel from Aquatic Species Project is to develop the technology base for large-scale production of oil-rich microalgae. The project is also developing methods to convert the microalgal lipids into liquid fuels needed for industry and transportation.

Project Objectives

Specific long-term objectives of the project are to

- Use genetic engineering as a tool to help control lipid accumulation

- Identify "trigger" points in biochemical pathways of algae that turn lipid production on and off

- Develop inexpensive, large-scale, outdoor mass culture technologies to grow microalgae

- Evaluate resource requirements for large-scale production of oil from microalgae as well as the environmental impact of such activities in the U.S. desert Southwest

- Develop technologies for converting microalgal lipids into valuable liquid transportation fuels, particularly biodiesel

- Transfer the technologies to the private sector for continued development and rapid commercialization by involving industry in the research process as early as possible.

Description of the Biodiesel from Aquatic Species Project Elements

Production

The NREL culture collection contains microalgal strains that produce large amounts of lipids and grow rapidly, but not necessarily in the same growth phase or in a controllable manner. Genetic improvement will be necessary to develop strains with the characteristics necessary to meet the overall project technical and economic goals, including predictable growth performance and controllable lipid yield. The genetic engineering process requires methods for getting genes (DNA) out of cells (cloning), modifying the genes, and reintroducing them into the microalgae. The modified genes would then confer one or more desired characteristics to the algae.

Extraction and Conversion

Methods need to be developed for economical extraction of lipids from microalgae and conversion of lipids to fuel substitutes. Untreated lipids have oxygen contents and viscosities that are too high to be used in standard engines. The primary goals of the conversion studies are to develop methods to economically convert a high proportion of the microalgal lipids to biodiesel fuels and to improve the overall economics by converting the balance of the biomass to biogas or other high-energy products.

Engineering Design

The technology to produce economic liquid fuels from microalgae will require the growth of microalgae on a large scale. Systems to maintain optimal levels of nutrients, carbon dioxide, salinity, and temperature must be developed and tested. The goal of the engineering design element is to develop large-scale outdoor facilities that allow the production goals to be met and to reduce the costs of such a system to those targeted by the project's economic analysis. It is envisioned that future work in this area will allow the evaluation of various mass-culture and harvesting systems and technologies in an effort to increase outdoor algal productivities and decrease the cost of operating such a facility.

Analysis

Economic and resource analyses provide input to project management in order for research directions and priorities to be set. The goal of the analysis element is to support the technology development by determining cost goals, economic sensitivities, resource assessments, and environmental impacts as new data are developed. To accomplish this, researchers will conduct ongoing economic analyses. Resource and environmental assessments will be conducted to identify potential constraints, identify and address data gaps, and provide project guidance.

Project Highlights

FY 1993 Accomplishments

The primary area of research during FY 1993 was the effort to genetically improve microalgae in order to control the timing and magnitude of lipid accumulation. Increased lipid content will have a direct effect on fuel price, and the control of lipid content is a major project goal.

Two major accomplishments in FY 1993 were the isolation of fragments of two microalgal genes:

nitrate reductase (NR) and acetolactate synthase (ALS). NR is an enzyme important in the partitioning of photosynthetic carbon products among lipids and other cellular components; understanding the genetics and biochemistry of this enzyme may lead to methods for controlling lipid production. ALS is an essential enzyme involved in amino acid biosynthesis. Modified forms of the ALS gene are responsible for conferring resistance to sulfonylurea herbicides; it was demonstrated during FY 1993 that at least one microalgal strain of interest to the project is sensitive to such herbicides. Both the NR and ALS genes will be useful as selectable markers for microalgal transformation. Using the polymerase chain reaction (PCR), fragments of both of these genes have been isolated from the green alga *Monoraphidium minutum*. These products will be valuable in isolating of the full-length versions of these genes from a previously-constructed genomic library.

The gene encoding the enzyme acetyl-CoA carboxylase (ACC) was isolated and characterized from *Cyclotella cryptica* previously. ACC appears to play an important role in controlling the levels of lipids accumulated in microalgal cells. Research in FY 1993 was directed at manipulation of the gene into a form that can be used more readily in other organisms in order to assess the effects of ACC overexpression. A manuscript detailing the gene isolation and characterization work was published (Roessler and Ohlrogge 1993).

Work on a method of introducing DNA into algal cells using silicon carbide fibers was published during FY 1993 (Dunahay 1993).

FY 1994 Plans

Genetic engineering will continue to be a major focus of the project. This area has the potential to have a positive effect on product yield. In FY 1994, research will focus on further manipulation and expression studies with the ACC gene from *C. cryptica* and on the cloning of the ALS and NR genes for use as selectable markers for microalgal transformation. Efforts will be directed at application of the newly generated gene probes to genomic libraries for isolation of the full-length genes. Work will also continue on refining the methods for physically introducing genes into microalgal cells.

II. CULTURE COLLECTION

The microalgal culture collection was transferred to fresh culture medium quarterly during FY 1993, and the comprehensive culture collection data base was updated. There is a total of 431 strains of algae in the collection: 34 in the cold water collection, 80 in the clone collection, and 317 in the main collection. During the past year only one strain failed successful transfer, which is an excellent record for a collection of this size. Substitution of autoclavable culture media for media that requires filter sterilization has allowed the complete elimination of some media types, thus streamlining the process of culture collection transfer.

III. MOLECULAR BIOLOGY OF LIPID BIOSYNTHESIS

INTRODUCTION

To genetically engineer an organism, such as a microalga, to carry out a particular function (e.g., lipid accumulation) better, the DNA composition of the organism must be altered by manipulating the genes that encode proteins of regulatory significance. A gene can be thought of as a stretch of DNA that gives an organism the ability to perform a specific function. For most genes, the DNA is "transcribed" into messenger RNA, which is then "translated" into a protein. In many cases, the protein product is an enzyme that catalyzes a particular chemical reaction.

Acetyl-CoA carboxylase (ACCase) is an enzyme believed to play a key role in controlling the rate of lipid biosynthesis in many organisms. This biotin-containing enzyme catalyzes the carboxylation of acetyl-CoA to form malonyl-CoA, which is the primary building block of fatty acids. This reaction has been shown to be highly regulated and is probably the rate-limiting step in fatty acid biosynthesis in animals (Kim et al. 1989; Lane et al. 1974). In addition, recent evidence suggests that differences in the rates of fatty acid biosynthesis in plants may be attributable to changes in ACCase activity (Post-Beittenmiller et al. 1991, 1992). Furthermore, past research at NREL has suggested a role for ACCase in the accumulation of storage lipids in the diatom *C. cryptica* in response to nutrient deficiency (Roessler 1988). As a consequence of these findings, ACCase has been receiving an increasing amount of attention as a target for manipulation via genetic engineering for the purpose of improving the lipid production capabilities of various organisms.

As reported previously, NREL researchers have cloned the ACCase gene from *C. cryptica*, representing the first time that this gene has been cloned from a photosynthetic organism. A manuscript describing the cloning and characterization of the ACCase gene was published during FY 1993 (Roessler and Ohlrogge 1993). Characterization work on the ACCase gene was continued during this period, and the gene was inserted into bacterial DNA vectors to allow introduction and overexpression of ACCase in other organisms, including the yeast *Saccharomyces cerevisiae.*

OVERVIEW OF ACCase GENE STRUCTURE

The complete DNA sequence of the ACCase gene from *C. cryptica* has been determined, and is scheduled for publication in the GenBank genetic data base (accession number L20784). In addition, the sequences of several regions of the ACCase gene transcript (i.e., messenger RNA) have been determined, which has allowed identification of the probable translation initiation codon, along with the precise localization of two introns in the gene. Introns, or intervening sequences, are nucleic acid segments that are spliced out of the RNA transcript of a gene. The presence of introns has implications for determining the correct amino acid sequence of the protein and therefore affects gene manipulation strategies. A 73-bp intron is present near the region of the gene that encodes the biotin binding site of the enzyme, and a 447-bp intron is located near the start of the coding region of the gene. Upon removal of these introns, a 6.3 kb coding sequence is obtained which encodes a protein that contains 2089 amino acids and has a molecular mass of approximately 230 kilodaltons. *C. cryptica* ACCase is therefore comparable in size to the multifunctional ACCases that have been isolated from various plants, animals, and yeast. The gene is large because ACCase contains three functional domains responsible for the different partial reactions involved in malonyl-CoA synthesis: biotin carboxylase, biotin carboxyl carrier protein, and carboxyltransferase.

Comparison of the deduced amino acid sequences of ACCase from *C. cryptica*, yeast, and rat indicated strong similarities in the biotin carboxylase and carboxyltransferase domains (~50% identity) but less similarity in the middle region of the enzyme (~30% identity). This middle region, which includes the biotin carboxyl carrier protein domain, probably functions as a "swinging arm" to transfer the carboxylated biotin from the biotin carboxylase domain to the carboxyltransferase domain. As such, a fair amount of variability in the amino acid sequence of this region could be tolerated, thus explaining the comparatively lower sequence conservation. Regions with statistically significant similarity are shown in Fig. 2, which is the graphical output generated by the MACAW sequence alignment computer program developed by Schuler et al. (1991).

In *E. coli*, ACCase is composed of four distinct, separable polypeptide components: biotin carboxylase, biotin carboxyl carrier protein, and two subunits of carboxyltransferase. Regions of the carboxyltransferase subunit from *E. coli* ACCase that are proposed to be involved in acetyl-CoA binding and carboxybiotin binding have been identified (Li and Cronan 1992a). The putative ATP-binding site of the biotin carboxylase subunit has also been designated (Li and Cronan 1992b). Comparisons of these regions with the corresponding regions of the multifunctional ACCases from *C. cryptica*, yeast (Al-Feel et al. 1992), and rat (Lopez-Casillas et al. 1988) are shown in Fig. 3.

The amino-terminal sequence of the predicted ACCase polypeptide, shown below using the standard one-letter amino acid code, has characteristics of a "signal sequence" (von Heijne 1990). Note the two positively-charged arginine (R) residues within the first few amino acids of the polypeptide, followed by a hydrophobic region:

MALRRGLYAAAATAILVTASVTAFAPQHSTFTPQSLSAAP. . .

Signal sequences direct polypeptides into the endoplasmic reticulum (ER) in eukaryotic cells. In diatoms, nuclear-encoded proteins destined for chloroplasts must be transported through the ER (Bhaya and Grossman 1991), which is consistent with the fact that diatom chloroplasts are completely enclosed by ER membranes (Gibbs 1979). Because fatty acid biosynthesis occurs primarily in the chloroplasts of higher plants (Harwood 1988), it is assumed that ACCase is located in the chloroplasts of diatoms, and therefore a signal sequence would be necessary for chloroplast targeting. Alternatively, it is possible that the ACCase gene that we have cloned codes for an ER-localized ACCase that is responsible for producing the malonyl-CoA used for elongation of fatty acids from C_{16} or C_{18} to C_{20} and C_{22}. Because ACCase must pass through the ER before it can enter the chloroplast, it is also possible that a single ACCase isoform is functional in both of these cellular compartments. Further experimentation will be necessary to examine this possibility. The location of the cleavage site at the end of the putative ACCase signal sequence is not clear; attempts to determine the amino-terminal sequence of the mature enzyme have not been successful. As discussed below, the presence of this signal sequence is relevant to the development of strategies for gene manipulation and expression.

ACCase GENE CONSTRUCTS

Construction of an Intron-Free Gene

Work during FY 1993 included efforts to produce a version of the ACCase gene for use in the genetic engineering of microalgae and other organisms for enhanced lipid production. The elimination of introns is one of the steps necessary before a gene can be expressed in heterologous host organisms, since the requirement for proper *in vivo* intron excision in the host organism would be eliminated. The large size of the ACCase gene precluded the isolation of a full-length complementary DNA (cDNA) clone in which the introns would already have been removed by natural processes. Therefore, an approach was taken to replace intron-containing regions of the gene with short cDNA fragments produced via the polymerase chain reaction process (Saiki et al. 1988; Lee and Caskey 1990; Frohman et al. 1988), using intron-free RNA as the starting template. In this way, portions of the genomic clone that did not contain introns could still be used. Unique restriction enzyme recognition sites that flanked the introns were used as the splice junctions for fragment replacement. The sequence information described above was critical to the success of this effort.

Construction of Expression Plasmids

Work is in progress to place the intron-free gene constructs into plasmid vectors that will allow for ACCase expression in bacteria, yeast, and plants. The primary rationale for inserting the ACCase gene into foreign hosts is that it will allow us to study the effects of overexpression of this gene. One strategy for increasing lipid production within a cell, or possibly for affecting the types of lipids produced, is simply to increase the levels of this rate-limiting enzyme. In other words, simply having more copies of the ACCase gene within the cell might have the effect of increasing the flow of carbon into lipids. There are two problems with doing such experiments directly in *C. cryptica*: 1) methods have not yet been developed to introduce DNA into this species (see discussion of genetic transformation below); and 2) effective overexpression can be more difficult in the organism from which the gene was isolated, because of inherent regulatory mechanisms that might reduce transcriptional expression to compensate for the extra number of gene copies. Overexpression of the gene in *C. cryptica* could also result in downregulation at the post-translational level, including possible covalent modification of the ACCase enzyme (e.g., phosphorylation/dephosphorylation). Introducing the gene into heterologous organisms such as yeast and plants should be relatively straightforward and should circumvent some of the potential problems outlined above.

Initial experiments are being directed at overexpression of ACCase in the yeast *Saccharomyces cerevisiae*. The intron-free *C. cryptica* gene has been inserted into commercially available yeast expression plasmids, and these plasmids have been introduced into yeast cells. Experiments are currently in progress to determine whether *C. cryptica* ACCase is being produced by the recombinant yeast, whether the enzyme is being properly biotinylated by the endogenous biotinylation system of *S. cerevisiae*, and whether an increase in lipid biosynthesis rates occurs.

We have also produced specific point mutations in the ACCase gene to facilitate cloning the gene into other expression vectors. For example, we have removed two restriction enzyme recognition sites (*Nco*I and *Xho*I) that were present in the coding sequence of the gene. This will simplify attachment of specific promoter and leader sequences to the 5' end of the gene and will allow efficient subcloning into certain expression vectors.

The yeast expression studies are an important step in the verification of the ACCase clone and in understanding the effects of ACCase overexpression in a simple biological system. Ultimately, however, it will be desirable to study the effects of expressing the *C. cryptica* gene in a photosynthetic cell. We have obtained modest funding through the NREL Director's Development Fund to attempt to produce transgenic higher plants containing the *C. cryptica* ACCase gene. The plant *Arabidopsis thaliana* will be used for these studies because it is one of the best model plant systems available. Efforts will be made to specifically express the ACCase gene in developing seeds in an attempt to increase seed oil accumulation.

In the long term, modification and expression of the ACCase gene may lead to significant improvements in lipid production by microalgae. It should be pointed out also that important commercial spinoffs may be produced along the way. For example, the plant expression studies described above could lead to methods for increasing the lipid contents of oilseed crops. Again, the *C. cryptica* gene may be particularly desirable for such efforts because of potential downregulation problems with native plant genes. Large markets exist for oilseed lipids, and improvements in lipid production could have a positive influence on oilseeds as a source of biodiesel, thus helping to establish the biodiesel market.

IV. MICROALGAL TRANSFORMATION

INTRODUCTION

Genetic transformation is the process of introducing DNA into a cell and having a gene contained on that DNA be expressed (read by the cell's biochemical machinery) to make a protein. The ability to "transform" an organism is central to any genetic engineering effort. To verify transformation, a method must be devised to detect the protein product biochemically or to apply a genetic selection such that only cells producing that protein are able to reproduce. Thus, transformation not only requires the physical introduction of DNA into the cell, but also requires a means of monitoring gene entry and expression. In addition, the creation of stably transformed cell lines requires that the DNA become fixed into the cell's genome.

Research in microalgal transformation has focused primarily on methods to assess the successful introduction of foreign DNA. A significant effort has been devoted to the development of appropriate selectable markers for microalgae that will provide a powerful selection for rare, stable transformation events. Testing of DNA introduction methods has also been a major focus.

HOMOLOGOUS SELECTABLE MARKERS

We are currently working to develop two genes as potential selectable markers for algal transformation. Our DNA analysis data (Jarvis et al. 1992), and the precedent set in *C. reinhardtii*, suggest that heterologous selectable markers (e.g., antibiotic resistance genes from bacteria) may not be effective in many of the algae. By using homologous genes, problems in promoter specificity and codon bias will not inhibit gene expression, allowing successful selection for transformed cells.

Nitrate Reductase (NR)

The NR gene has been used successfully as a transformation marker in a number of organisms, including fungi (Daboussi et al. 1989) and *C. reinhardtii* (Kindle et al. 1989). Using NR as a selectable marker requires isolation of the wild-type gene and the generation of NR mutant cells. Algal cell mutants lacking functional NR can be selected based on their resistance to chlorate. Cells having a functional NR protein will take up chlorate along with nitrate and reduce it to chlorite, which is toxic to the cells. The NR-minus mutants do not reduce the chlorate and therefore do not experience the cytotoxic effects of chlorite. Consequently, cells can be subjected to a positive selection regime by growing target organisms in the presence of chlorate and picking out resistant colonies. Using this protocol, we previously isolated several putative NR-deficient mutants of *M. minutum*; these candidates grow in the presence of chlorate and are unable to use nitrate on plates or in liquid culture. Initial biochemical characterization of these mutants, in conjunction with growth studies, indicated that at least two of the mutant strains contain lesions in the NR structural gene and would thus be appropriate targets for transformation with the homologous wild-type gene. Further characterization of these mutants is still in progress.

Experiments in FY 1993 have been aimed primarily at the cloning of the wild-type NR gene from *M. minutum*. Although NR genes from other algae, such as *C. reinhardtii*, may be able to complement NR mutations in our strains of interest, the likelihood of achieving transformation would be much greater if the homologous genes could be isolated. In other words, transforming a mutant of *M. minutum* with a gene from *M. minutum* has a much better chance of success; this is expected because the gene from *M. minutum* carries all of the correct signals to be read by the algal cell. Once the gene is cloned, complementation of an NR-minus mutant with a functional NR gene will result in cells that can use nitrate as the sole nitrogen source, providing a powerful selection for transformants.

Attempts to clone the *M. minutum* NR gene have focused on the use of PCR with primers based on NR gene sequences from related organisms. This approach proved to be successful during FY 1993. Amino acid sequence information is available for NR proteins from a number of higher plants and at least three eukaryotic green algae. Comparison of these sequences indicated several regions of very high sequence similarity between all species examined. A set of PCR primers was designed based on the NR sequences from the green algae *C. reinhardtii*, *Volvox carteri*, and *Chlorella vulgaris* and from several higher plants including tobacco, tomato, corn, and *Arabidopsis*. As a control, the primers were tested initially in PCR reactions using genomic DNA from *C. reinhardtii* as a template. Reaction conditions were found that produced a single PCR product of the predicted size of approximately 790 bp. Similar conditions were then used in PCR reactions with *M. minutum* genomic DNA as a template, producing a single band of approximately 750 bp. The PCR product from *M. minutum* was cloned and partially sequenced. The *M. minutum* sequence showed significant homology to the NR sequences from the other green algae, indicating that the product was derived from NR sequences.

The PCR experiments were successful in isolating a fragment of the NR gene from *M. minutum*. For use in transformation, the gene must be cloned in its entirety. To this end, the PCR gene fragment can be used as a probe to identify lambda clones from a *M. minutum* gene library containing the full-length gene. The first step in this approach, the generation of the library, was accomplished during FY 1992. A genomic library contains the entire genome (DNA content) of an organism broken down into smaller pieces; these pieces are then incorporated into the genome of viruses (such as the bacteriophage lambda) so that the incorporated foreign genes are naturally amplified. This library of cloned DNA pieces is then screened with a labeled probe, such as the NR PCR product, thereby allowing the investigator to identify and isolate the desired gene. DNA libraries were created in two similar lambda phage vectors from different commercial sources, λGEM12 (Promega) and λFIX2 (Stratagene), using genomic DNA from *M. minutum* that was partially cleaved with the restriction enzyme *Sau*3A. Combined, the libraries contain approximately 340,000 separate clones. Each phage should not be viable unless it has received an insert of at least 9,000 bp. Assuming a DNA content of under 5×10^7 bases per cell, this implies that the libraries should contain multiple copies of each gene in the organism. Recent analysis of several individual recombinants from both libraries shows actual insert sizes of 14,000 to 18,000 bp.

We are currently attempting to screen the libraries for full length NR gene sequences using the 750 bp PCR product as a probe. The DNA fragment is labeled with digoxigenin-dUTP via PCR or the random priming reaction and hybridized to lambda phage DNA that has been transferred to a nylon filter. Positive plaques are detected using the Genius™ Non-Radioactive Detection System (Boehringer Mannheim Biochemicals). The initial screen has yielded only what appeared to be false positives; i.e., lambda plaques were identified that hybridized well with the PCR probe but that failed to pass further tests. This work is ongoing. Once true NR-containing phage have been isolated, the inserts will be mapped, subcloned, and partially sequenced. Plasmids containing the full-length NR gene will then be used in transformation experiments with the putative NR mutants of *M. minutum*.

Orotidine-5'-phosphate Decarboxylase (OPD)

The OPD gene codes for an essential enzyme in the pyrimidine biosynthetic pathway. OPD mutants grow only if provided with an alternate source of pyrimidines, such as uracil. There is also a positive selection for OPD mutants, which involves using the drug 5-fluoroorotic acid (FOA). The OPD gene has been cloned and sequenced from a number of organisms (e.g., Rose et al. 1984; Newbury et al. 1986; Ohmstede et al. 1986; Turnbough et al. 1987), and has been used successfully in several transformation systems (e.g., Buxton and Radford 1983; Boy-Marcotte et al. 1984; van Hartingsveldt et al. 1987). Previous work using the FOA mutant selection method allowed us to obtain several putative OPD mutants from UV-mutagenized cells of the green alga *M. minutum*. These cells require uracil for growth, thus allowing a powerful selection for cells that have been transformed with the wild-type OPD gene. The next step in the development of this system is the isolation of the wild-type algal OPD gene. Several approaches to cloning the *M. minutum* OPD gene were attempted previously, either based on PCR methodologies or on complementation of OPD mutants of bacteria. To date, these approaches have failed to yield the OPD gene. For this reason, further efforts will be directed primarily at the development of other selectable marker systems.

Acetolactate Synthase (ALS)

Work began in FY 1993 on the development of a new selectable marker system that has a great deal of potential for microalgal transformation. ALS is an enzyme involved in the biosynthesis of branched-chain amino acids (leucine, isoleucine, and valine). This enzyme is sensitive to a number of sulfonylurea and imidazolinone herbicides, which act as competitive inhibitors (Babczinski and Zelinski 1991; Schloss 1990). Altered forms of this enzyme (the products of mutated ALS genes) are responsible for resistance to such herbicides (Friedberg and Seijffers 1990; Sathasivan et al. 1990). Such mutated ALS genes can therefore be used as selectable markers based on their ability to confer herbicide resistance.

Using herbicide resistance as a selectable marker has the advantages of traditional antibiotic selections in that resistance genes can be used as dominant selectable markers. This means that wild-type organisms can be transformed directly without the need to first generate mutants (as described above for NR). The process of generating mutants can be particularly problematical in diploid cells such as *C. cryptica* since two genes must be altered simultaneously. In addition, if herbicide resistance genes are isolated from the organism to be transformed, homologous selectable marker systems can be developed. This requires cloning and invitro mutation of the wild-type herbicide-sensitive gene or cloning of the gene from mutated organisms that show resistance (Fig. 4). Again, using a homologous system ensures that lack of expression resulting from improper codon bias or promoter sequences will not be a problem. Herbicide resistance genes that have been used in plant transformation (reviewed in Botterman & Leemans 1988) include 5-enol-pyruvylshikimate-3-phosphate synthase (EPSPS; responsible for glyphosate resistance) and ALS. The ALS system has recently been applied to the production of transgenic rice (Li et al. 1992).

Thus, herbicide resistance genes have potential as selectable markers for the transformation of microalgae. In the past, several species in the NREL culture collection, including *M. minutum* and *C. cryptica*, have been shown to be sensitive to a number of common herbicides (Galloway 1990). During FY 1993 these experiments have been repeated and expanded. Levels of the sulfonylurea herbicides chlorsulfuron, and sulfometural methyl that inhibit the growth of *M. minutum* have been determined through cell plating assays. Levels as low as 1.5μg/mL sulfometuron methyl completely inhibited growth of this alga. This sensitivity will allow a positive selection for transformed cells once an appropriate form of the ALS gene is in hand. Unfortunately, these two herbicides were not shown to inhibit the growth of *C. cryptica* at the levels tested. Experiments are in progress to determine if this poses an insurmountable barrier to the use of ALS as a marker gene in this diatom. Alternatively, very recent experiments indicate that growth of *C. cryptica* is completely inhibited in the presence of 1 mM glyphosate, suggesting that the EPSPS gene may be a good choice as a selectable marker for this diatom.

Significant progress was made during this review period in the cloning of the ALS gene from *M. minutum*. Degenerate PCR primers were designed based on comparisons of published ALS sequences from plants and yeast. In PCR experiments, a specific fragment of DNA from *M. minutum* was amplified. This was achieved using two sets of nested primers in sequential reactions. The primary product was cloned into a plasmid vector and sequenced. The deduced peptide sequence of this short (approximately 125 amino acid) segment showed 46% and 36% amino acid identity to the published sequences of the ALS genes from a plant (*Arabidopsis*) and a red alga (*Porphyra*), respectively. This suggests that a segment of the ALS gene has in fact been cloned from *M. minutum* that can be used to obtain the full-length clone from our gene library (as described above for NR). Interestingly, a second gene fragment cloned at the same time is closely related in sequence, suggesting the presence of an isozyme or pseudogene for ALS in this alga. Initial attempts to isolate the full-length ALS clone have so far failed to yield positive results.

DNA INTRODUCTION METHODS

Once suitable selectable marker systems have been devised, the main barrier to transformation becomes the physical introduction of DNA into the algal cell. The presence of well-developed cell walls in most microalgae may make this a difficult problem to overcome as protocols for preparation of protoplasts are unavailable for most species. Several methods have proved successful in the transformation of other organisms. Our initial transformation experiments will focus on two methods: silicon carbide and biolistics.

Silicon Carbide

Results were reported in FY 1992 on the use of silicon carbide (SiC) for the transformation of a test alga, *Chlamydomonas reinhardtii.* In this work, a published method of agitating plant suspension culture cells in the presence of DNA and SiC whiskers (Kaeppler et al. 1990, 1992; Asano et al. 1991) was applied to algal cells for the first time. The protocol for transformation of *C. reinhardtii* cells using SiC whiskers is relatively straightforward. An NR-deficient strain of *C. reinhardtii* (*nit1-305 mt*) was used as the recipient strain, and the plasmid used to complement the NR deficiency contained a genomic DNA insert encoding the full-length sequence for the NR gene from *C. reinhardtii.* Cells in late exponential or early stationary growth phase were harvested and placed into a small volume of fresh medium. Plasmid DNA, polyethylene glycol (PEG), and a slurry of SiC whiskers were added to each tube. The samples were then agitated vigorously using a laboratory mixer, after which the cells were spread onto petri plates containing selective medium (nitrate being the sole nitrogen source). Transformants appeared within 10 to 14 days. Transformation was confirmed by Southern analysis, which demonstrated the presence of vector DNA sequences and additional randomly inserted copies of the NR gene within the recipient cell genome. A manuscript describing this work was published recently in the journal *Biotechniques* (Dunahay 1993).

Stable nuclear transformation of walled *C. reinhardtii* cells was achieved by this method at a rate of 10 to 100 transformants per 10^7 cells. This is comparable to the efficiency obtained with a published protocol (Kindle 1990) using glass beads instead of SiC whiskers. However, SiC vortexing results in very little cell death and therefore appears to be a more gentle procedure. Glass beads (200-300 μm) are much larger than algal cells; glass bead-mediated transformation apparently works by physically damaging the cell wall and membrane, often resulting in cell death. The SiC fibers may work by nicking or puncturing the wall and/or membrane, allowing DNA entry through a less severe wound. It is hoped that the SiC method will prove to be a generally applicable method for the introduction of DNA into other algal species that have greater potential for lipid production in outdoor mass culture.

Biolistics

The only other available method for reproducibly introducing DNA into walled plant or algal cells is microprojectile bombardment or biolistics. In this technique, helium pressure is used to propel DNA-coated gold or tungsten particles (microprojectiles) into target cells. Biolistics has been used successfully to transform a wide variety of plants, animals, and microorganisms (Klein et al. 1992; Smith et al. 1992), including walled cells of the green alga *C. reinhardtii*. We will be using the PDS-1000/He Particle Delivery System from Bio-Rad Laboratories to attempt to introduce DNA into microalgae once the marker expression systems have been developed.

V. ENVIRONMENT, SAFETY, AND HEALTH AND QUALITY ASSURANCE

During FY 1993 the standard of excellence in environment, safety, and health (ES&H) was maintained. Because of these efforts, routine laboratory inspections surfaced only very minor problems, and these were dealt with promptly.

The inventory of chemicals in the laboratories was updated on a monthly basis during FY 1993. The inventory, which can be accessed through the laboratory computer, includes information such as order and receipt dates and particular hazards associated with a chemical. Laboratory-specific chemical hygiene plans, safe operating procedures, and similar documents were written or updated for each of the Biodiesel from Aquatic Species Project laboratories.

The calibration of equipment within the laboratories was maintained according to schedule.

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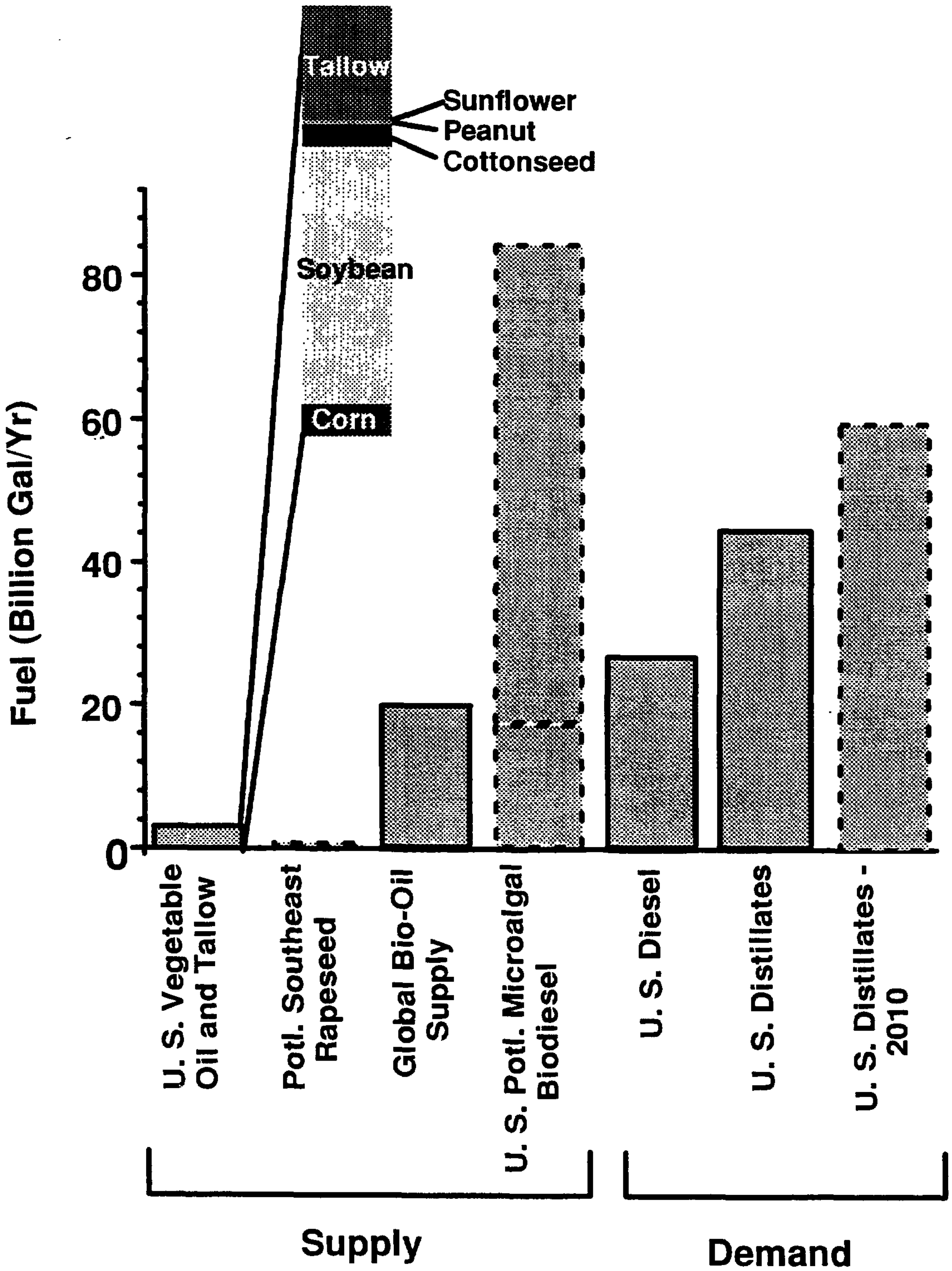

Figure 1. Vegetable oil, tallow, and microalgal biodiesel potential supplies compared to energy market sizes. Note that U. S. vegetable oil and tallow and global bio-oil supplies (vegetable, tallow and marine oils) are not sufficient to supply even the U. S. biodiesel market. Two estimates for microalgae are given; low estimate based on project target and high estimate based on carbon dioxide sources in southern U. S. (Feinberg and Karpuk 1990). Resource data are from Cambridge Energy Research Associates (1992), Commodity Research Bureau. (1992), Energy Information Administration (1993), Raymer and Van Dyne (1992b), United States Department of Agriculture. (1992). Dashed lines represent projections.

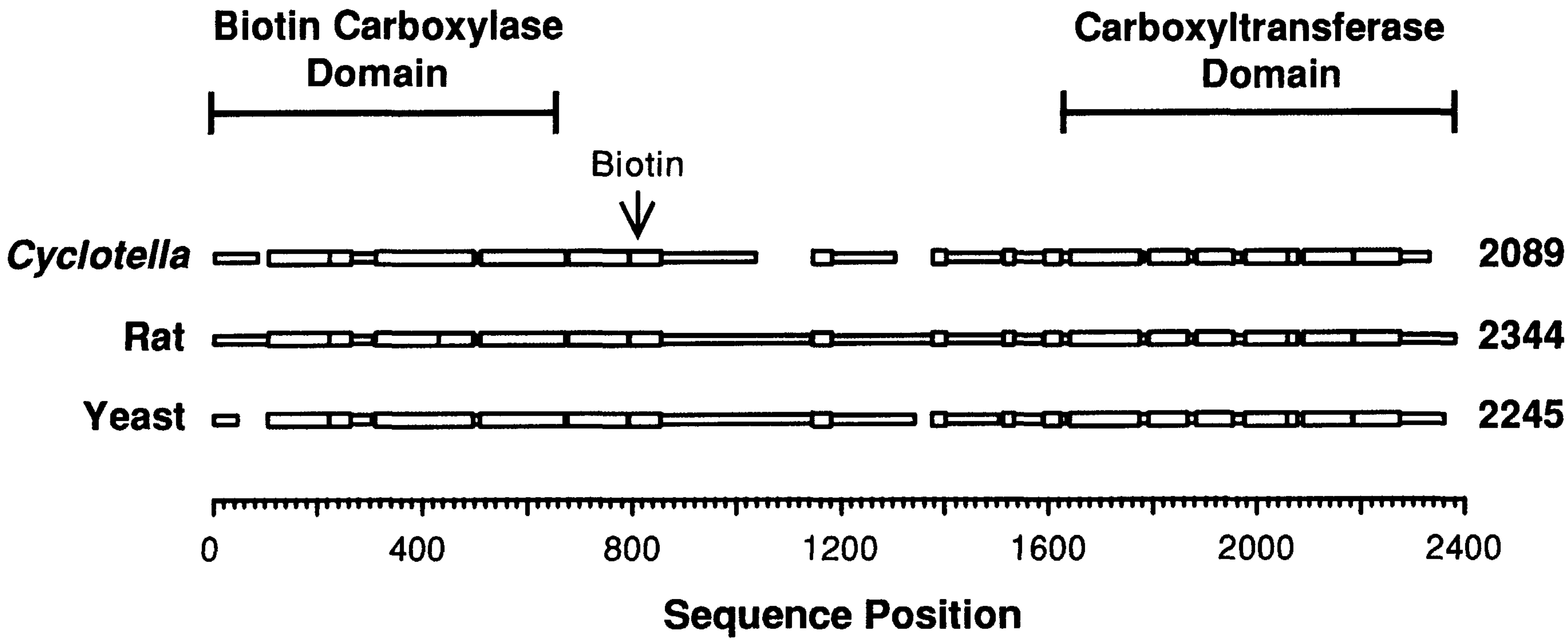

Figure 2. Schematic diagram indicating regions of similarity among the amino acid sequences of ACCase from *C. cryptica*, rat, and yeast. The MACAW program of Schuler et al. (1991) was used to align the sequences. Regions (> 19 amino acids) that are present in all three sequences and that exhibit statistically significant homology ($p < 0.000001$) are indicated by thickened areas.

A.

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Cyclo ACC (1476-1526)      G R Q V V V I V N D V T V Q S G S F G V E E D E V F F K A S K Y A R E N K L P R V Y I A C N S G A R I
Yeast ACC (1579-1629)      G R Q F V V V A N D I T F K I G S F G P Q E D E F F N K V T E Y A R K R G I P R I Y L A A N S G A R I
Rat ACC (1672-1722)        G R D V I V I G N D I T Y R I G S F G P Q E D L L F L R A S E L A R A E G I P R I Y V A A N S G A R I
E.coli β-CT (117-167)      G M P V V A A A F E F A F M G G S M G S V V G A R F V R A V E Q A L E D N C P L I C F S A S G G A R M
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B.

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Cyclo ACC (1758-1777)      G K S V V I G R G R L G G I P M G A I A
Yeast ACC (1878-1897)      A K G V V V G R A R L G G I P L G V I G
Rat ACC (1967-1986)        A Q T V V V G R A R L G G I P V G V V A
E.coli α-CT (98-117)       D K A I V G G I A R L D G R P V M I I G
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C.

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Cyclo ACC (287-307)        E N G I M I K A S E G G G G K G I R F V D
Yeast ACC (246-266)        G F P V M I K A S E G G G G K G I R Q V E
Rat ACC (304-324)          G Y P V M I K A S E G G G G K G I R K V N
E. coli BC (153-173)       G Y P V I I K A S G G G G G R G M R V V R
```

Figure 3. Alignment of amino acid sequences of ACCase from several sources in the regions proposed to be involved in the binding of A) carboxybiotin, B) acetyl-CoA, and C) ATP. Residues enclosed in boxes are identical to the sequence of *C. cryptica* ACCase. Abbreviations: α-CT, α subunit of carboxyltransferase; β-CT, β subunit of carboxyltransferase; BC, biotin carboxylase.

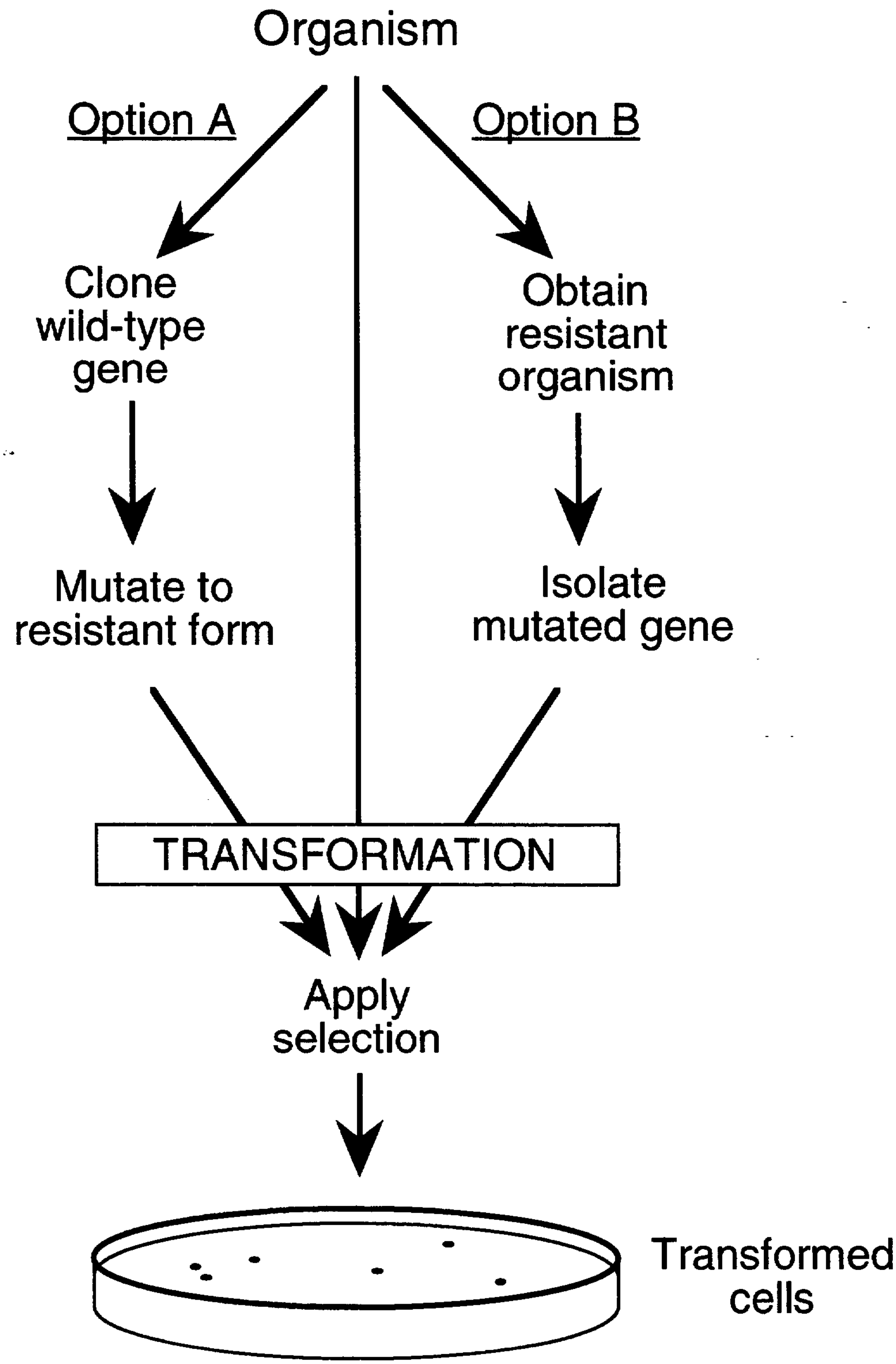

Figure 4. Development of transformation systems using dominant homologous selectable markers. The marker gene is generated either through the cloning and alteration of the wild-type gene (Option A) or by cloning the gene from an organism exhibiting resistance to the selective agent (Option B). Once obtained, the marker gene can then be transferred into the wild-type organism. Application of selection then allows only transformed cells to grow.

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15. Supplementary Notes

16. Abstract (Limit: 200 words)

This report summarizes the progress and research accomplishments of the Biodiesel from Aquatic Species Project field managed by the National Renewable Energy Laboratory through 1993. The project, funded through the Biofuels Systems Division of the U.S. Department of Energy, focuses on using microalgae as a feedstock for producing renewable, high-energy liquid fuels. Its goal is to develop the technology base for large-scale production of oil-rich microalgae. In addition, the project is developing methods to convert microalgal lipids into liquid fuels needed by industry and for transportation. The primary area of research during FY 1993 was the genetic improvement of microalgae to control the timing and magnitude of lipid accumulation.

17. Document Analysis

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